Cooperative strand displacement by peptide nucleic acid (PNA)

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Background: Synthetic homopyrimidine peptide nucleic acids (PNAs) can bind complementary targets in double-stranded DNA, generating strand-displacement complexes, and so offering an opportunity to modulate specific gene expression. Several issues remain to be addressed before these attributes can be exploited *in vivo*, however.

Results: The kinetics of the interaction between a homopyrimidine PNA and a complementary homopurine target on double-stranded DNA were analyzed in the presence or absence of a preformed strand-displacement complex proximal to the target. The complex was established under low salt conditions by the binding of a different homopyrimidine PNA to a target situated adjacent to the first PNA target. These two targets were placed next to each other on opposite strands at distances of 0, 2, 4 and 8 base pairs apart. The presence of a preformed strand-displacement complex near the target accelerates the binding of PNA to double-stranded DNA in a salt-dependent manner. The influence of salt on the binding rates was also examined. The binding rate is increased by a factor of $1 \times \exp(70[\mathrm{NaCl}])$, that is, 16-fold at 40 mM NaCl and more than 10^4-fold if extrapolated to 140 mM NaCl. This effect is significantly reduced if the two targets are 2 base pairs apart and completely absent if the distance is 4 base pairs or more.

Conclusions: The perturbation of the DNA helix imposed by a PNA strand-displacement complex only propagates a few base pairs. It is therefore possible to target sites in the immediate vicinity of strand invasion complexes specifically. The results presented have implications for the mechanism of strand displacement and for the application of PNA in a genomic context.

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Introduction

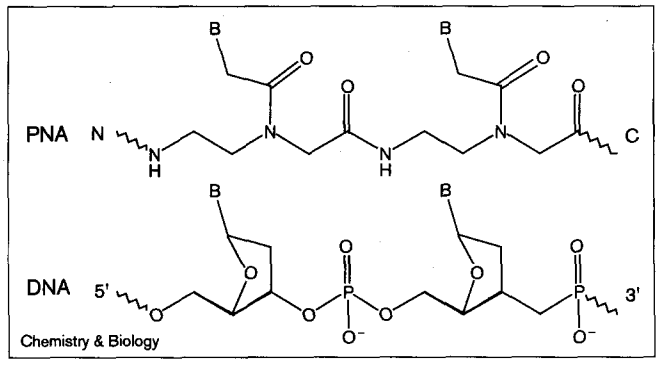
Peptide nucleic acid (PNA) is a synthetic DNA mimic in which the sugar–phosphate backbone has been replaced by a structurally homomorphous and uncharged pseudopeptide backbone to which the nucleobases are attached via methylene carbonyl linkers (Figure 1) [1]. PNAs containing pyrimidines have been found to bind complementary targets of double-stranded (ds) DNA by a mechanism termed strand displacement, which leads to formation of PNA_2–dsDNA complexes [2]. The two homopyrimidine PNA strands form Watson–Crick and Hoogsteen hydrogen bonds, respectively, with the complementary homopurine DNA strand, resulting in a PNA_2–DNA triplex with remarkably high stability. Consequently, the homopyrimidine DNA strand is displaced by the PNA and left as a virtually single-stranded loop [1–3].

When targeting the template strand of a transcribed sequence, a PNA_2–DNA complex is sufficiently stable to arrest transcriptional elongation [4–10]. PNA therefore is a good candidate for sequence-specific modulation of gene expression employing an 'anti-gene' strategy [11]; there are several potential obstacles for using PNA as a gene-targeted drug *in vivo*, however. One of these is the effect of physiological salt levels on PNA binding: although strand-displacement complexes remain stable at least up to 500 mM NaCl [3], the rate of complex formation is severely reduced at elevated ionic conditions [2–5,12–15].

The mechanism of strand-displacement complex formation is not yet fully understood. A kinetic model of strand displacement which suggests that a transient opening of the DNA duplex within the PNA target is a rate-limiting step has been proposed [13]. This notion is compatible with the fact that cations reduce the rate of PNA strand displacement, possibly by stabilizing the DNA duplex and so reducing the probability of transient openings or other distortions facilitating strand displacement. Stabilization of the DNA double helix by cations, therefore, may be considered a rate-limiting factor for strand-displacement complex formation.

We speculated that binding of a PNA targeted to a dsDNA site that was kept partially open would be less susceptible to changes in ionic conditions. Permanganate probing of strand-displacement complexes previously revealed not only that thymines facing the PNA target but also those thymines flanking the target were accessible for oxidation [16]. This indicated that the structure of

Figure 1



The chemical structure of PNA compared to that of single-stranded DNA. B indicates a nucleobase.

the DNA helix in the immediate vicinity of the PNA_2–DNA triplex is distorted — partially open.

Here, we have investigated the binding characteristics of a PNA targeted to a dsDNA site kept partially open by a second strand-displacement complex situated in the immediate vicinity of the dsDNA site of interest. The data presented here indicate that binding of a PNA to such a partially open target is dramatically enhanced at physiologically relevant ionic strengths, and is less sensitive to changes in salt concentration compared to binding of PNA to a native target. The findings are compatible with the hypothesis that DNA duplex stability is a rate-limiting factor for strand invasion by PNAs at elevated ionic strengths, and have implications for the application of PNA in a genomic context. Furthermore, the results show that the helix distortion is only 'sensed' a few base pairs away from the 'lesion'.

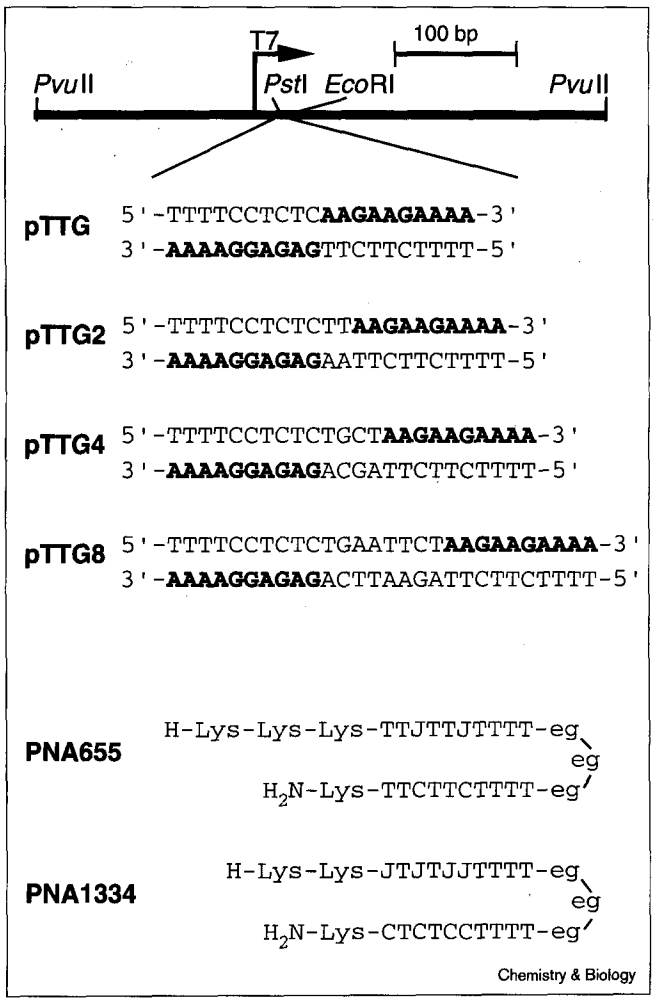
Results

Binding of PNA1334 (Figure 2) to DNA constructs possessing separate PNA targets for PNA655 and PNA1334 was monitored using a gel mobility shift assay [4,13]. Four different DNA constructs, denoted pTTG, pTTG2, pTTG4, and pTTG8, have two PNA targets each, separated by 0, 2, 4 and 8 base pairs, respectively (Figure 2). Binding of PNA1334 to the DNA fragments gave rise to a shift in mobility, indicating formation of a PNA–DNA complex (Figure 3). When PNA655 was prebound to the target fragments, binding of PNA1334 gave rise to an additional shift in mobility (Figure 4).

PNA1334 binding to the native pTTG fragment

Binding of PNA1334 to the native target was examined under various salt concentrations (Figure 3a). The fast-migrating species corresponds to the unbound DNA fragment and the slow-migrating species corresponds to the PNA1334–dsDNA complex. To analyze the binding kinetics, the fractions of unbound DNA (D) were determined

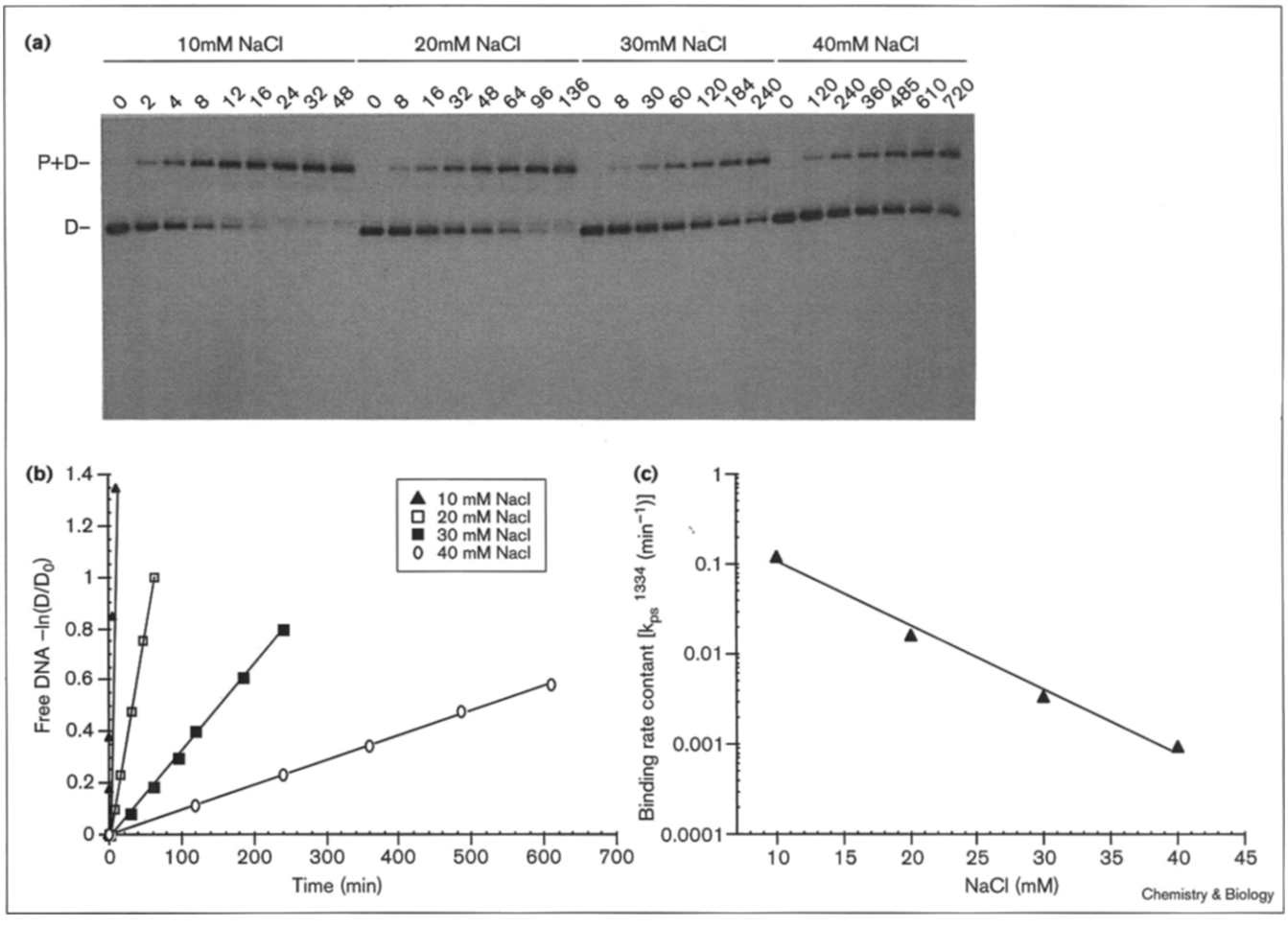
Figure 2



Structure of the DNA constructs and the PNAs we used. **(a)** The DNA constructs were 0.47 kb *Pvu*II fragments isolated from pBluescript KS^+ derivatives. The plasmids denoted pTTG, pTTG2, pTTG4, and pTTG8 were made by inserting targets (boxed) for PNA1334 (lower strands) and PNA 655 (upper strands) separated by 0, 2, 4, and 8 base pairs, respectively, into the polylinker of pBluescript KS^+ (see Materials and methods section for details). **(b)** The structures of the bis-PNAs PNA655 and PNA1334. J and eg designate the synthetic nucleobase pseudoisocytosine and ethylene glycol linker units, respectively [20].

by scanning densitometry. By plotting the natural log of D against time it was found that binding of PNA1334 to the pTTG target obeyed pseudo-first-order kinetics (Figure 3b) and the binding rate constant k_{ps}^{1334} was dramatically reduced as the ionic strength was increased (Figure 3c). The binding rate as a function of the NaCl concentration was found to fit the expression $k_{ps}^{1334} = 0.7 \exp(-160\,[NaCl])$. In control experiments, PNA1334 was incubated with a DNA fragment containing a target for PNA655 only, or the pTTG fragment was exposed to a nonrelated PNA; in both cases, no binding was observed (data not shown).

Figure 3



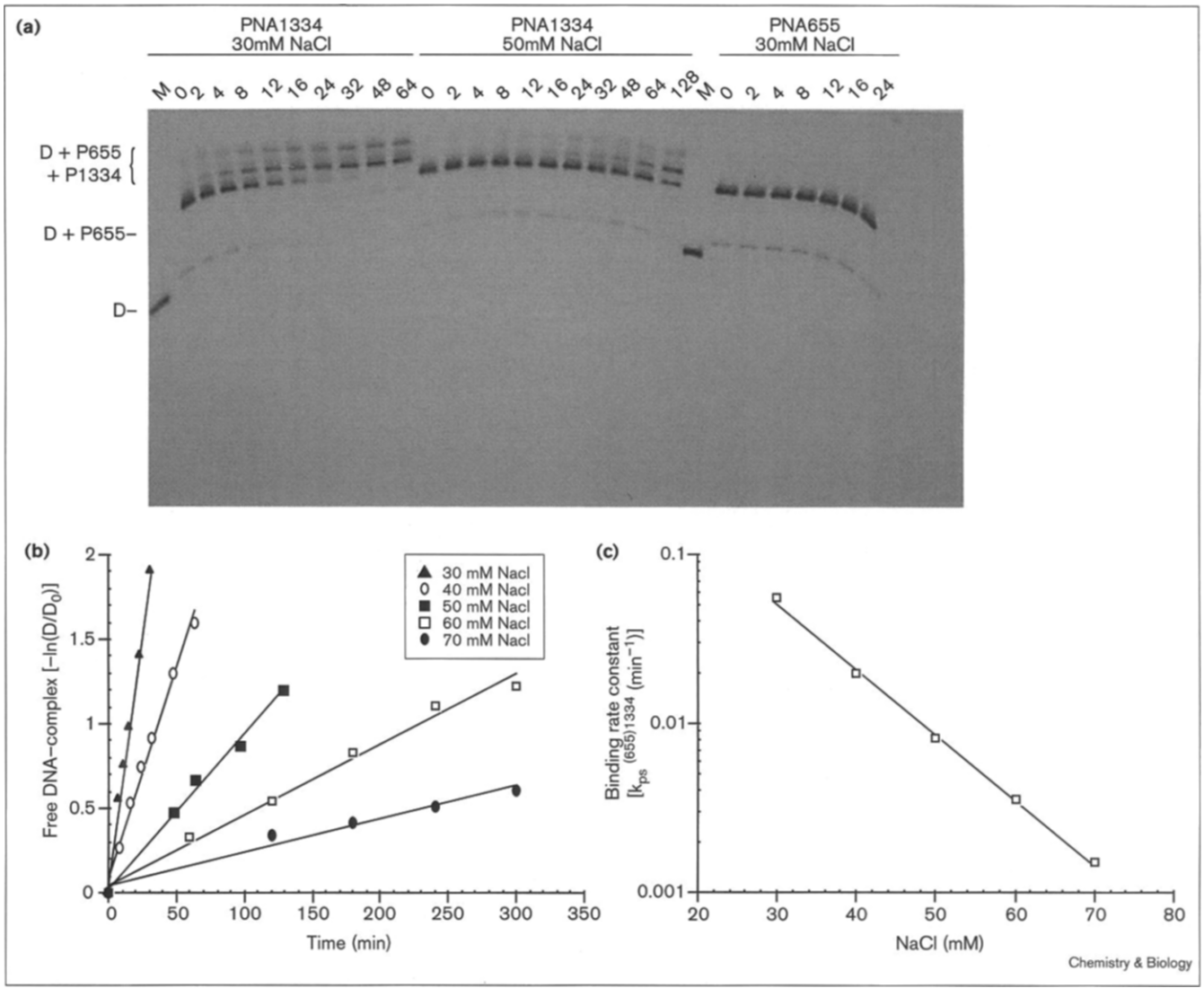
Kinetics of PNA1334 binding to the native pTTG/*Pvu*II DNA fragment under various ionic conditions. **(a)** A typical gel shift experiment in which binding of PNA1334 to pTTG is monitored. The ionic conditions and the length of incubation (minutes) are indicated above each lane and the origins of the different species are indicated to the left: PNA1334–DNA complex, P+D; free DNA, D. The species migrating slightly slower than the free DNA is of unknown origin but appears inert with respect to PNA binding. **(b)** Analysis of the pseudo-first-order kinetics at different concentrations of NaCl. The negative natural logarithm to the fractions of free DNA (D/D_0) is plotted as a function of incubation time. **(c)** Dependence of the pseudo-first-order rate constant on the salt concentration.

Binding of PNA1334 to the pTTG fragment with a preformed PNA655 strand-displacement complex

To investigate the kinetics of PNA binding to a dsDNA target kept partially open, PNA655 was used to form a strand-displacement complex with the pTTG fragment in the immediate vicinity of the PNA1334 target. After appropriate preincubation of the pTTG fragment with PNA655 (see the Materials and methods section), virtually all of the DNA molecules were converted to strand-displacement complexes (Figure 4a). Following adjustment of the ionic strength to the desired value, PNA1334 was added and binding was followed as a function of time. Interestingly, binding of PNA1334 to the pTTG fragment already involved in a PNA655 strand displacement complex near the target resulted in not one but two supershifted bands (Figure 4a). Because these two bands appear with similarly slow kinetics, they were both assumed to be strand-displacement complexes, probably corresponding to two structurally distinct complexes (possibly resulting from interference between the multiple lysine residues attached to the PNA termini; see Figure 2). Band migration could also depend on what side the ethylene glycol linker of the PNA passes the DNA backbone of the target strand. To ensure that the PNAs bound their cognate targets only, the end-labeled *Pvu*II fragment of pTTG was probed with $KMnO_4$ in the presence of either or both PNAs. No strand displacement could be observed beyond the relevant PNA targets (data not shown).

Kinetic constants were derived essentially in the same way as for PNA1334 binding alone, except that in this case exponential decay of the band corresponding to the pTTG

Figure 4



Kinetics of binding of PNA1334 to the pTTG *Pvu*II fragment containing prebound PNA655. **(a)** Gel-shift experiment in which binding of PNA1334 to the pTTG fragment prebound to PNA655 is monitored at various concentrations of NaCl. The NaCl concentration and the length of incubation (in minutes) are shown above each lane. Free DNA was applied as a marker to lanes named M. The origins of the different species are indicated to the left (PNA1334–PNA655–DNA complex, D + P655 + P1334; PNA655–DNA complex, D + P655, and free DNA, D). None of the bands migrates as pTTG in complex with PNA1334 only, that is, binding of PNA1334 does not promote dissociation of PNA655 (data not shown). The species migrating slightly slower than the free DNA is of unknown origin but appears inert with respect to PNA binding. **(b)** Analysis of the pseudo-first-order kinetics at different concentrations of NaCl. The negative natural logarithm to the fractions of free DNA–PNA655 complex is plotted as a function of incubation time. **(c)** Dependence of the pseudo-first-order rate constant on the NaCl concentration.

fragment with prebound PNA655 was followed over time. Figure 4b shows that binding of PNA1334 to the DNA fragment with prebound PNA655 was consistent with pseudo-first-order kinetics, but the binding rates were significantly higher than those obtained for PNA1334 binding to the native pTTG fragment. For instance, at 40 mM NaCl the rate was accelerated by one order of magnitude. Furthermore, from a plot of the binding rates $k_{ps}^{(655)1334}$ against the concentration of NaCl (Figure 4c), it is clear that the salt dependence of the binding rate in this case is less pronounced (exponent power –90) than that observed for PNA1334 binding to its undistorted native target. To ensure that the PNA655 still present in solution during PNA1334 binding did not cross-react with the target for PNA1334, a control experiment in which a further amount PNA655 was added instead of PNA1334 was conducted (Figure 4a). No significant cross-reaction occurred under the conditions employed.

Partial opening of the dsDNA target brought about by a preformed strand-displacement complex in the immediate vicinity of the PNA target site dramatically enhanced the PNA binding rate and significantly reduced the inhibitory effect of salt on strand displacement. We use the thermodynamic term 'cooperativity' to describe this phenomenon, although we have measured only the on-rate. There is no reason, however, to expect that the off-rate is affected by the binding of a 'helper-PNA' and therefore the on-rate alone should directly reflect the affinity of the PNA for its target.

Cooperativity of PNA binding operates over only a short distance

To determine the distance at which a strand-displacement complex can affect the binding rate to another target, the binding of PNA1334 to a target placed 2, 4, or 8 base pairs away from a preformed PNA655–dsDNA complex was analyzed using the pTTG2, pTTG4, and pTTG8 *Pvu*II fragments, respectively (Figure 2). The results, presented in Figure 5, show that the cooperativity was highly dependent on the distance between the two PNA targets. If the two targets were situated only 2 base pairs apart, the cooperativity was significantly reduced compared to the situation described above where the targets were situated immediately next to each other. Increasing the distance to 4 base pairs resulted in complete absence of cooperativity, that is, the binding rate was similar to that obtained for the native PNA1334 target. Thus cooperativity of PNA strand displacement is restricted to targets spanning the regions in the immediate vicinity of a preformed strand-displacement complex.

To examine whether an increased rate of PNA binding could be correlated with increased accessibility of the thymines in and near the target, the different DNA fragments complexed with PNA655 were probed with $KMnO_4$ (Figure 5c). As indicated in Figure 5d, the distortion of the PNA1334 target caused by PNA655 is dramatic for pTTG, less significant for pTTG2, and below the level of detection for pTTG4 and pTTG8. Thus, there is a good correlation between the degree of cooperativity and the PNA655-mediated opening of the PNA1334 target.

'Strand-switch' targeting

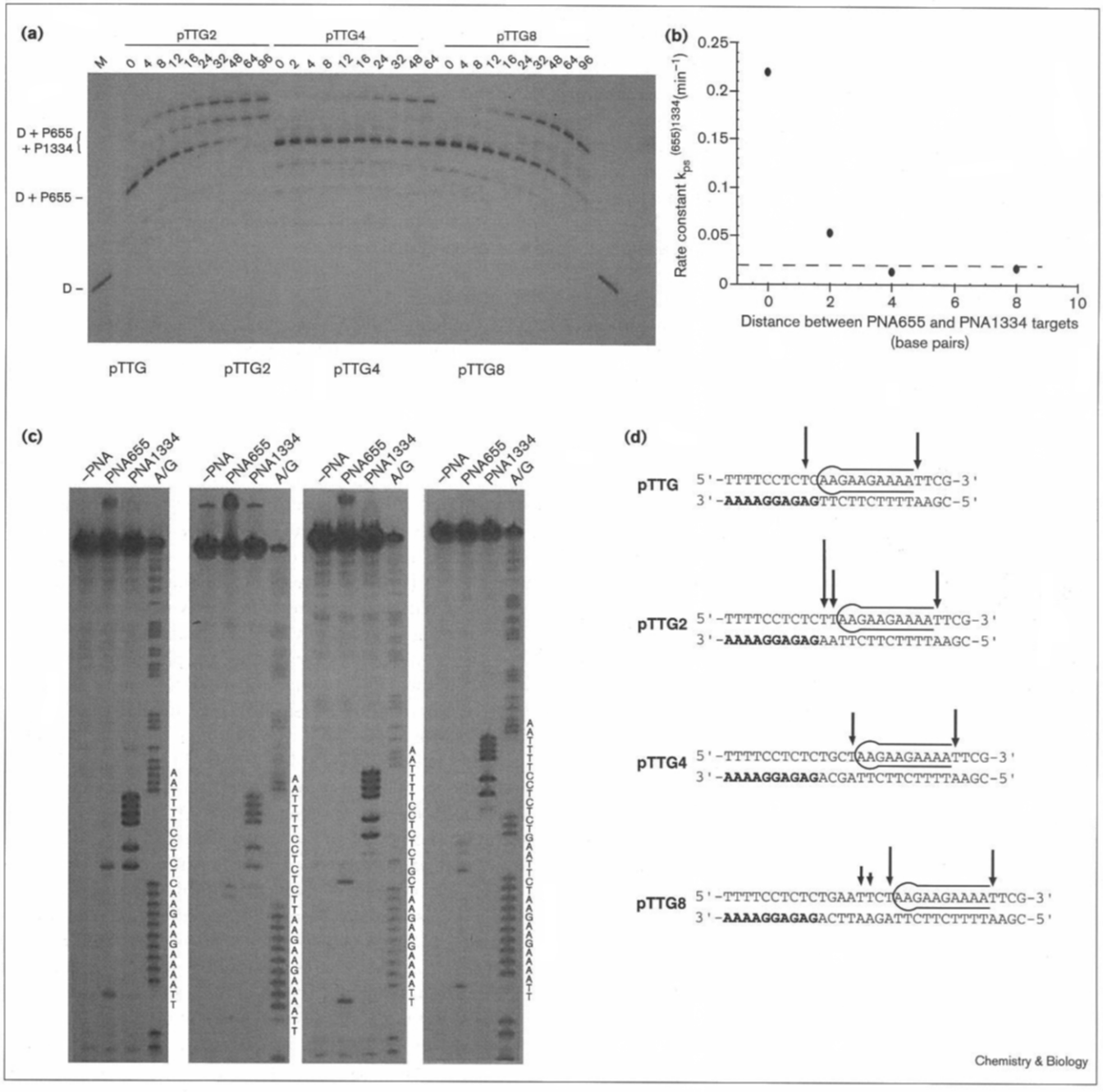
Cooperative action of two (different) homopyrimidine PNAs targeted to adjacent sequences on opposite strands might be exploited for 'strand-switch' targeting. This could be suitable in cases where one of the strands is accessible, but the other strand is the important one to target in order to get an 'anti-gene effect', that is transcriptional arrest. Also, cooperative action would increase the sequence specificity when targeting longer DNA sequences. To test this, the *Pvu*II fragment of pTTG (or the same fragment in complex with PNA655) was exposed to various concentrations of PNA1334 in the presence of 70 mM NaCl and used as a template for T7 RNA polymerase *in vitro* transcription (Figure 6). Because the target for PNA655 in this setup is situated on the non-template strand, binding of PNA655 alone, as expected, did not result in transcriptional arrest [4,8,9]. Binding of PNA1334 to the template strand, in contrast, is expected to result in transcriptional arrest. But PNA1334 alone did not bind to the template efficiently under the given conditions. Only when both PNAs were present was efficient transcriptional arrest observed. Thus, the presence of a transcriptionally 'inert' PNA can mediate the binding of a second 'effector' PNA, resulting in transcriptional arrest.

Discussion

DNA duplex stability is generally believed to be the main rate-limiting factor for PNA strand-displacement at elevated salt concentrations. This is consistent with the following observations. Triplex formation occurs rapidly and essentially independent of salt concentration, at least up to 150 mM of monovalent cations, when the homopurine target is single stranded (H.J.L. and P.E.N., unpublished observations). Also, the activation energy for strand displacement has been found to be of the same order of magnitude as that required for opening of a few base pairs in duplex DNA [15], and a transient opening of a DNA duplex brought about by a passing RNA polymerase accelerates PNA binding dramatically [9]. Furthermore, binding of PNA is significantly facilitated at physiological ionic strength if the DNA containing the PNA target is under negative supercoiling stress [15]; and finally, the rate of PNA binding increases if the non-target strand has mismatches, nicks or gaps (H.J.L. and P.E.N., unpublished observations). Thus, any factor that provides partial or complete DNA duplex opening of a PNA target appears to accelerate the rate of strand displacement. The results presented here are fully compatible with this notion, as binding of PNA was enhanced dramatically if it was targeted to a site in the immediate vicinity of a preformed strand-displacement complex. This cooperativity of PNA binding was dependent on the salt concentration in an exponential manner. Prebinding of PNA655 in the immediate vicinity of the PNA1334 target increased the rate of binding by a factor of $1 \times \exp(70[\mathrm{NaCl}])$. This indicates that the presence of a preformed strand-displacement complex in the immediate vicinity of another target results in a fourfold increase in binding efficiency at 20 mM NaCl, a 16-fold increase at 40 mM NaCl, and more than 10,000-fold if extrapolated to physiological ionic strength, that is, 140 mM NaCl.

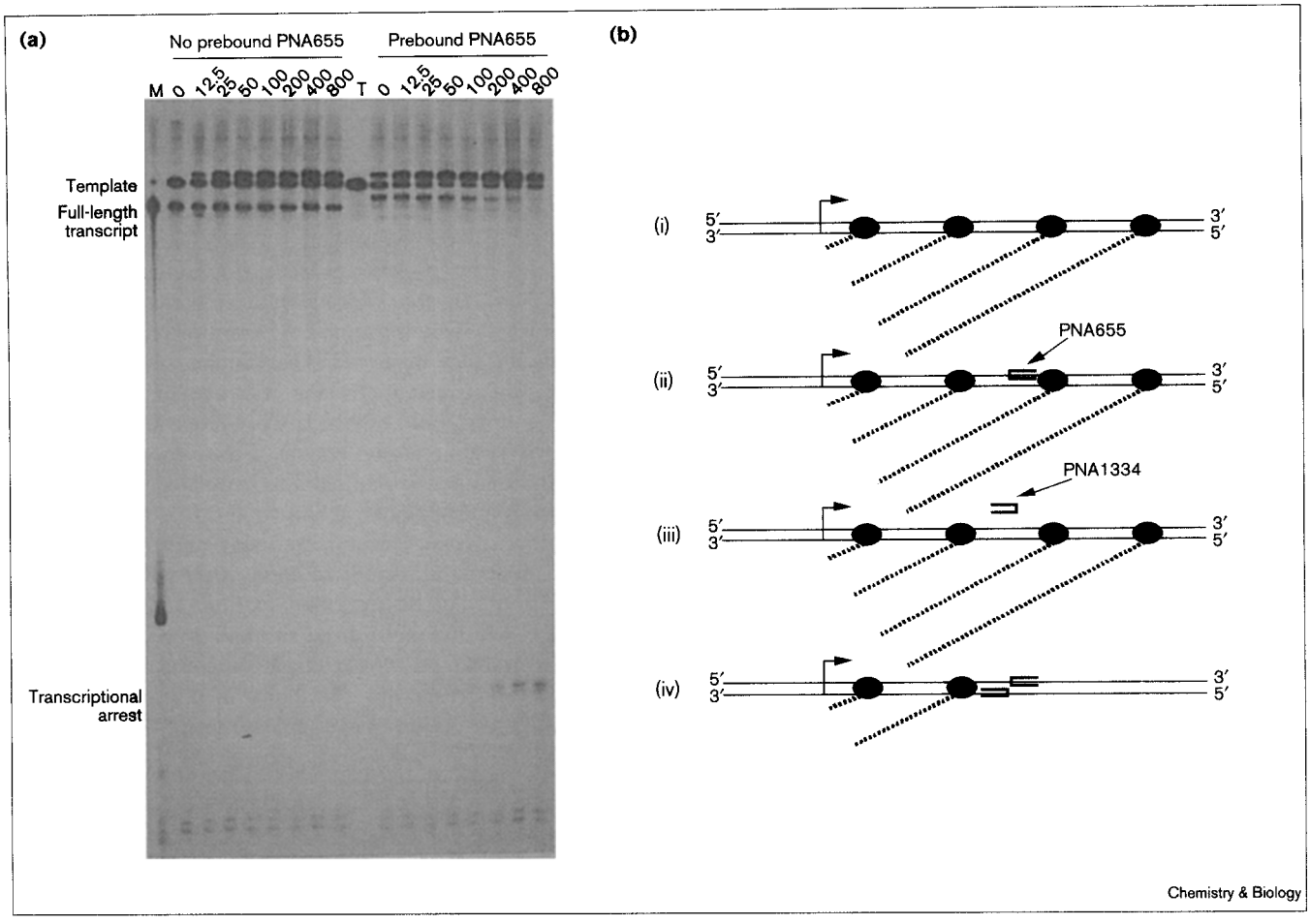
In addition to strand displacement by PNA, other naturally occurring events such as transcriptional initiation, replication, and recombination are known to be associated with distortions of the DNA duplex. It therefore seems reasonable to anticipate that these basic biochemical mechanisms could facilitate PNA strand displacement under physiological ionic conditions and thus improve

Figure 5



PNA1334 binding to the PNA655–pTTG2, PNA655–pTTG4, and PNA655–pTTG8 at 20 mM NaCl. **(a)** Results of a gel-shift assay. The origin and time of incubation (in minutes) are indicated above each lane. The composition of the complexes is indicated to the left (PNA1334–PNA655–DNA complex, D + P655 + P1334; PNA655–DNA complex, D + P655, and free DNA, D). **(b)** The pseudo-first-order rate constants as function of the distance (in base pairs) between the targets. The broken line indicates the value of k_{ps}^{1334} under similar conditions. **(c)** Detection of PNA655-mediated opening of the PNA1334 target by $KMnO_4$ probing. The *Sal*I–*Sac*I DNA fragments of pTTG, pTTG2, pTTG4, and pTTG8 were labeled at the *Sal*I site using Klenow DNA polymerase and [α-^{32}P]-dATP. This results in a 3′ end labeling of the strand containing a target for PNA655. The labeled DNA fragments were complexed with PNA655 or PNA1334 and single-stranded thymines were detected by their ability to react with $KMnO_4$. A/G sequence reactions were run in parallel in order to assign the bands. The DNA fragment and the PNA used are indicated above the lanes. The sequences of the PNA target regions are shown to the right for each panel. **(d)** The quantified results of the $KMnO_4$-probing experiments. The accessibility of thymines of the PNA655 target strand is illustrated by arrows. The lengths of the arrows reflect the reactivity of the individual thymines. The reactivity of the thymine immediately 3′ to the PNA655 target was used as an internal standard.

Figure 6



Transcriptional arrest brought about by cooperative action of two different PNAs. **(a)** The end-labeled *Pvu*II fragment of pTTG (or the same fragment in complex with PNA655) was exposed to various concentrations of PNA1334 in the presence of 70 mM NaCl and used as a template for T7 RNA polymerase *in vitro* transcription involving [α-^{32}P]GTP. The presence and absence of PNA655 and the concentration of PNA1334 are indicated above each lane. A molecular size marker prepared by T7 polymerase *in vitro* transcription of pTTG/*Pvu*II, pBluescript KS$^+$/*Hin*dIII, pBluescript KS$^+$/*Bam* HI resulting in radiolabeled transcripts of 355 nucleotides (nt), 81 nt, and 52 nt, respectively, was run in parallel (M). In the lane marked T, the radiolabeled template alone has been applied. The positions of the template, the full-length transcript, and the truncated transcript resulting from transcriptional arrest are indicated; the predicted length of the truncated transcript is 67 nt. Because the truncated transcript is much shorter than the full-length run-off transcript and consequently contains fewer labeled guanosines it results in a weaker signal. The presence of a complementary PNA reduces the migration of a fraction of some of the species. **(b)** A cartoon explaining the experiment shown in (a). (i) Normal transcription. (ii) Transcription is unaffected by PNA655 bound to the non-template strand. (iii) PNA1334 alone did not bind efficiently the template under the given conditions. (iv) Only when both PNAs were present was efficient transcriptional arrest observed.

the prospects for the possible future use of PNA as an 'anti-gene drug' operating *in vivo* to suppress pathological gene transcription.

By determining the rates of PNA binding to targets situated 2–8 base pairs away from the preformed complex, we conclude that cooperativity of PNA strand displacement is restricted to targets spanning the regions in the immediate vicinity of a preformed strand-displacement complex. This is consistent with results from experiments in which PNA_2–dsDNA complexes were chemically probed with $KMnO_4$, indicating that the distortion of the DNA duplex caused by a strand displacement complex is propagated only a few base pairs away, into the DNA flanking the PNA target. Similar results were obtained for a duplex-forming strand-displacing homopurine PNA [16]. Furthermore, PNA_2–dsDNA complexes have the capacity to inhibit restriction enzyme cleavage of DNA [12]. Consistent with the results reported here, such PNA-mediated inhibition of restriction enzyme cleavage is relieved if the enzyme and PNA recognition sites are situated more than 5 base pairs apart [12]. Finally, it has been estimated that at temperatures below the DNA helix–coil transition, the probability of a single base-pair opening is as low as 10^{-5} for a base pair

inside the helix and 0.1 for a base pair at the edge of the helix [17]. This is consistent with NMR studies of the 'end' effects on base-pair opening in B-DNA duplexes, which showed that the dissociation constant of a base pair may be affected by 'end effects' up to the second, or at most the third, base pair from the end itself [18]. These data correlate very well with our results which show that the binding of a second PNA is unaffected if its target is only 4 base pairs away and only moderately facilitated when the targets are separated by 2 base pairs.

If PNA is to be developed as an anti-gene therapeutic agent, several issues have to be addressed. Statistically, PNA should be capable of discriminating between a single mismatch and a perfect match within a 15–16-mer sequence — essential in order to minimize the possibility of inhibiting the 'wrong genes'. It is reasonable to assume, however, that the effects of single mismatches will decrease with increasing length of the PNA. It might therefore not be appropriate to employ long PNAs. Alternatively, cooperative PNA binding could be exploited to limit the number of targets to those situated immediately adjacent to a 'helper' target. Ideally, complexes formed at such helper targets would not, by themselves, have sufficient stability for transcriptional arrest, but would only catalyze the binding of a second and longer 'effector' PNA, eventually leading to complexes with the desired anti-gene activity.

It is possible to identify conditions under which a PNA will bind only if its target is in close proximity to a strand-displacement complex. This was illustrated by 'strand-switch' experiments that led to transcriptional arrest only if both PNAs were involved. This approach could be highly relevant for the possible future development of PNA as a gene-targeted drug. For instance, we showed that binding of PNA mediated by the passing transcription elongation complex occurred more efficiently when the PNA target was placed on the non-template strand, but transcription is blocked efficiently only if the PNA is targeted to the template strand [4,8,9]. A similar approach could also be relevant for the use of PNA in genome mapping. It should be stressed, however, that the sequence restrictions of strand displacement have to be solved before these aspects of the results presented can be fully exploited.

Significance

Peptide nucleic acid (PNA) is a synthetic mimic of naturally occurring nucleic acids and is considered to be one of the prototypes for the possible future development of gene-targeted pharmaceuticals. Homopyrimidine PNAs can bind complementary targets in double-stranded DNA in a sequence-specific manner by a mechanism termed strand displacement, leading to PNA_2–dsDNA complexes. The rate of strand displacement is, however, dramatically reduced by the presence of physiological levels of salt. We have found that binding of homopyrimidine PNA to a complementary target in dsDNA is accelerated by the presence of a preformed strand-displacement complex in the immediate vicinity of the target. This cooperativity is highly dependent on ionic strength and on the distance between the target and the preformed strand displacement complex. Notably, the cooperativity of PNA binding was dependent on the salt concentration in an exponential manner. Prebinding of PNA655 close to the PNA1334 target increased the rate of binding by a factor of exp(70[NaCl]) corresponding to a factor of 16 at 40 mM NaCl or, by extrapolation, to a factor of 10^4 at 140 mM NaCl (a physiological sodium concentration). If the two targets were separated by 4 or more base pairs, no cooperativity was observed. Our findings have at least two potential implications for the use of PNA in a genomic context, both of which were illustrated by our *in vitro* transcription assay results. First, cooperativity of PNA binding may be exploited to 'transmit' the effect of PNA binding from one strand to the other, leading to transcription arrest. Second, by using appropriate PNA concentrations and sufficient ionic strength, this same phenomenon may be exploited to limit the number of effective PNA targets to those situated immediately adjacent to a preformed strand displacement complex — an approach to increase the 'specificity' of the second PNA, by limiting the number of available targets.

Materials and methods

PNAs

The bis-PNAs H-$(Lys)_3$-TTJTTJTTTT$(eg)_3$TTTTCTTCTT-Lys-NH_2 and H-$(Lys)_2$-JTJTJJTTTT$(eg)_3$TTTTCCTCTC-Lys-NH_2 denoted PNA655 and PNA1334, respectively, were synthesized and characterized as described previously [19,20]. J and eg indicate the synthetic nucleobase pseudoisocytosine and ethylene glycol (8-amino-3,6-dioxaoctanoic acid) linker units, respectively. The lysine residues attached to the PNAs increase their positive charge and thus the rate of complex formation (P.E.N. and V. Demidov, unpublished observations).

DNA constructs

All DNA manipulations were carried out using standard protocols [21]. Target plasmids pTTG, pTTG2, pTTG4 and pTTG8 that have 0, 2, 4 and 8 base pairs (bp) between their two PNA targets, respectively, were constructed by inserting the appropriate oligonucleotide pairs into the polylinker of plasmid pBluescript KS^+, using the recognition site(s) for *Eco*RI and *Pst*I as cloning sites (Figure 2). The sequences of the oligonucleotide pairs were: 5′-AATTTTCCTCTCAAGAAGAA-3′/3′-AAGGAGAGTTCTTCTTTTAA-5′ (pTTG0); 5′-CGAATTTTCCTCTCTTAAGAAGAA-3′/3′-ACGTGCTTAAAAGGAGAGAATTCTTCTTTTAA-5′, (pTTG2); 5′-CGAATTTTCCTCTCTGCTAAGAAGAA-3′/3′-ACGTGCTTAAAAGGAGAGACGATTCTTCTTTTAA-5′, (pTTG4); and 5′-CGAATTTTCCTCTCTGAATTCTAAGAAGAA-3′/3′-ACGTGCTTAAAAGGAGAGACTTAAGATTCTTCTTTTAA-5′ (pTTG8). The structures of all four constructs were verified by sequencing. Radiolabeled DNA fragments used in binding experiments were prepared by digestion of the relevant plasmids using *Pvu*II, dephosphorylation of the resulting fragments with alkaline phosphatase, and labeling with T_4 kinase and [γ-^{32}P] ATP. The radiolabelled 468 bp, 470 bp, 472 bp, and 476 bp *Pvu*II fragments of pTTG, pTTG2, pTTG4, and pTTG8 respectively (containing the PNA targets) were isolated on native 5% polyacrylamide gels.

Binding experiments

The relevant ^{32}P-labeled pTTG fragment (4 nM) was incubated at 37°C with 60 nM of PNA1334 in 100 µl of TE buffer (10 mM Tris–HCl pH 7.5,

1 mM EDTA, pH 8 at various concentrations of NaCl. Aliquots were withdrawn at different time points and the reaction was quenched by adding up to 50 mM salt and freezing on dry ice. For analysis of PNA binding to a site adjacent to a strand-displacement complex, the ^{32}P-labeled DNA fragment was preincubated with 50 nM of PNA655 at 37°C for 80 min in 100 µl of TE buffer (pH 7.5), and 10 mM NaCl. After establishment of the PNA655–DNA strand displacement complex, the NaCl concentration was adjusted to the desired value before the kinetics of PNA1334 binding was measured.

Binding of PNA1334 to its targets on dsDNA fragments was analyzed using a gel-mobility-shift assay. Using 10% polyacrylamide (TAE buffer) nondenaturing gels it was possible to resolve free DNA, PNA655–DNA complexes, and PNA1334–PNA655–DNA complexes. Quantification was carried out using autoradiography followed by densitometric analysis using a Molecular Dynamics computing densitometer and ImageQuant™ software.

Rate constant determination

Previously it was shown that that PNA binding to dsDNA is virtually irreversible and thus obeys pseudo-first-order kinetics when the concentration of PNA is in a large excess relative to the DNA target, that is, it can be considered constant during the reaction [13,14]. Consequently, strand displacement can be followed as exponential decay of unbound DNA over time:

$$D = e^{(-k_{ps}t)} \quad (1)$$

where D is the fraction of unbound DNA and k_{ps} is a pseudo-first-order rate constant. Plotting the negative natural logarithm to the fraction of free DNA as a function of incubation time yields a graph with the slope k_{ps}. The kinetics of PNA1334 binding to DNA fragments containing a strand-displacement complex was analyzed in the same way but in this case the exponential decay of prebound DNA was followed.

$KMnO_4$-probing of strand-displacement complexes

Ten µg of pTTG, pTTG2, pTTG4, and pTTG8 were digested with *Sal*I in total volumes of 50 µl. The linearized plasmids were 3′-end-radiolabeled by the addition of 3 µl of dGTP, dCTP, dTTP (500 µM each), 3 µl [α-^{32}P]-dATP (10 µCi/µl), and Klenow fragment of DNA polymerase (15 min at 0°C). The labeled DNA was purified by phenol–chloroform and chloroform extraction and recovered by ethanol precipitation. The DNA was redissolved and digested with *Sac*I in a total volume of 50 µl. The radiolabeled 0.1 kb *Sal*I–*Sac*I fragments of pTTG, pTTG2, pTTG4, and pTTG8 were isolated from a 5% native polyacrylamide gel. The isolated radiolabeled 0.1 kb *Sal*I–*Sac*I fragments of pTTG, pTTG2, pTTG4, and pTTG8 (~20 ng each) were incubated in low-salt buffer (10 mM NaCl, 10 mM Tris–HCl, pH 7; 1 mM EDTA) with PNA655 (50 nM) and/or PNA1334 (250 nM) in a total volume of 100 µl at 37°C for 1 h. The DNA was oxidized with 5 µl 20 mM $KMnO_4$ for 15 s and the reaction was stopped by adding 75 µl 1 M β-mercaptoethanol, 1.5 M NaOAc, pH 9. The oxidized DNA was ethanol-precipitated and cleaved in 100 µl 10% piperidine at 90°C for 20 min. Following lyophilization, the samples were run on a 10% polyacrylamide–7 M urea sequencing gel and analyzed by autoradiography. The bands resulting from reactions involving PNA655 were quantified using laser scanning densitometry and ImageQuant™ software. The cleavage of the thymine situated immediately 3′ relative to the PNA655 target was used as an internal standard.

PNA-mediated transcription arrest

Ten ng of the end-labeled *Pvu*II fragment of pTTG (or the same fragment in complex with PNA655) was subjected to various concentrations of PNA1334 in the presence of 70 mM NaCl, 10 mM Tris-HCl (pH 7), 1 mM EDTA, and 10 ng/µl pBluescript KS$^+$ linearized with *Bam* HI as a binding control for 1 h at 37°C in a total volume of 30 µl. *In vitro* transcription was carried out by mixing 20 µl of the binding reaction with 80 µl labeling mixture (50 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 2.5 mM spermidine–HCl, 31.25 mM NaCl, 12.5 mM DTT, 625 µM ATP, CTP, and UTP, 62.5 µM GTP, 0.03 µCi/µl [α-^{32}P]GTP, 0.5 U/µl RNase inhibitor, and 2.5 ng/µl pBluescript KS$^+$ linearized with *Not*I as a labeling control). The reactions were started by the addition of of 10 units T7 RNA polymerase, incubated for 4 min at 37°C, and stopped by adding 300 µl ethanol and 10 µl 4 M NaCl. The nucleic acids were recovered by centrifugation and analyzed using 10% polyacrylamide–7 M urea sequencing gels and autoradiography.

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